Colocalization of GABA-Immunoreactivity in Neuropeptide- and Monoamine-Containing Amacrine Cells in the Retina of *Bufo marinus*

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Summary. Immunocytochemical study was performed in the *Bufo marinus* retina to reveal the localization of y-aminobutyric acid (GABA)-immunoreactivity in neuropeptide Y (NPY)-, substance P (SP)-, serotonin (5HT)- and tyrosin hydroxylase (TH)-immunoreactive amacrine cells. GABA-immunoreactivity was present in all NPY-, in some of the SP- and 5HT-containing amacrine cells, but not in TH-immunoreactive amacrine cells. Among the 5HT-immunoreactive amacrine cells, a population of 5HT-synthesizing and most of the 5HT-accumulating cells were GABA-immunoreactive. These results indicate that neuropeptide- and GABA-immunoreactivity are colocalized in amacrine cells of the anuran retina. We propose a possible co-transmission for two classical neurotransmitters (GABA and 5HT) in some of the 5HT-containing amacrine cells.

Previous studies have indicated that the y-aminobutyric acid (GABA)-ergic amacrine cell population is morphologically (POURCHO and GOEBEL, 1983; YANG et al., 1991) and immunohistochemically (VERSAUX-BOTTERI et al., 1987; OSBORNE, 1988; POURCHO and GOEBEL, 1988; VANNEY and YOUNG, 1988a, b; WATT et al., 1988; YAZULLA and YANG, 1988; CASINI and BRECHA, 1992; WATT, 1992) heterogeneous in the vertebrate retina. Neuropeptides or monoamines that are colocalized in GABA-immunoreactive cells have been found to correspond to a few morphologically distinct amacrine cell types (MASSEY and REDBURN, 1987; VANNEY, 1990).

Recently we have reported that about 50% of the amacrine cells in the retina of the cane toad (*Bufo marinus*) are GABA-immunoreactive (GÁBRIEL et al., 1992a). However, the dendritic morphology of individual GABA-immunoreactive cell types could not be studied, due to dense labelling in the inner plexiform layer (IPL). In the past few years a number of wide-field amacrine cell types have been morphologically characterized in the *Bufo marinus* retina; these contain substance P (SP; HISCOCK and STRAZNICKY, 1989a), neuropeptide Y (NPY; HISCOCK and STRAZNICKY, 1989b), serotonin (5HT; ZHU and STRAZNICKY, 1990a) and tyrosine hydroxylase (TH), a marker for dopaminergic cells (ZHU and STRAZNICKY, 1990b). Of these putative neurotransmitters/modulators, SP (POURCHO and GOEBEL, 1988), TH (VERSAUX-BOTTERI et al., 1987; WASSLE and CHUN, 1988; WULLE and WAGNER, 1990) and serotonin or its preloaded analogs (OSBORNE, 1988; WATT, 1992) are colocalized with GABA in various amacrine cell populations. In contrast, no colocalization study has been carried out involving GABA and NPY.

In the present study we attempted to colocalize the above four markers in GABA-immunoreactive amacrine cells of the *Bufo marinus* retina.

**MATERIALS AND METHODS**

Adult cane toads (*Bufo marinus*) were obtained from a local supplier and kept under 12 h light-dark cycles. Animals were sacrificed by an overdose of tricaine methanesulphonate (Sigma), the eyes dissected from the orbit, the neural retina separated from the other coats of the eye ball in phosphate-buffered saline.

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and fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (pH 7.4) for 4–6 h at room temperature. After overnight washing in PBS, tissue pieces were cryoprotected in 30% sucrose-phosphate buffer and cross sections were cut in a cryostat at 14–18 μm. The sections were air-dried, then pretreated with an antibody diluent (1% ovalbumin, 0.1% Triton X-100, 0.1% NaN₃ in PB). The primary antibodies (Table 1) were applied in the following combinations: mouse anti-GABA together with antibodies raised in rabbits against TH, NPY, SP and 5HT. In addition, rabbit anti-GABA antibodies were applied together with monoclonal anti-phenylalanine hydroxylase (PH) raised in mice. The latter antibody, apart from phenylalanine hydroxylase which is not present in nervous tissue, recognizes tryptophan 5-hydroxylase and tyrosine hydroxylase (HAAN et al., 1987). Thus with some precaution, this antibody can be used as a marker for 5HT-synthesizing cells in the anuran retina (ZHU et al., 1992). The primary antibodies were applied overnight at room temperature, the sections then being washed in PBS. Fluorochrome-coupled secondary antibodies, goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; Seralab) and horse anti-mouse IgG coupled to Texas Red (TR; Seralab), were applied for 2 h at room temperature. Sections were then washed in PBS, mounted in bicarbonate-buffered glycerol (pH 8.6), viewed and photographed in a Leitz Orthoplan microscope.

Omission of primary antibodies from the incubation steps or their replacement with non-immune sera eliminated any staining. Preabsorption of the primary antibody in GABA-glutaraldehyde-BSA complex overnight at 4°C completely abolished GABA-labelling, while preabsorption with other amino acid conjugates (glutamate, aspartate, alanine, glycine and taurine) did not affect the immunoreaction. Preabsorption controls for the peptide antigens (HISCOCK and STRAZNICKY, 1989a, b) and control labelling experiments in the brain stem with the antibodies raised against TH and PH (WILHELM et al., 1993) have already been described. Cross-reactivities between non-corresponding primary and secondary antibodies as well as leakage of non-specific wavelength light through the microscope filters were tested according to previous descriptions (ZHU et al., 1992). The labelling pattern achieved by the mouse and rabbit anti-GABA antibodies were directly compared, revealing that they labelled the same structures in the retina of *Bufo marinus*.

### RESULTS

Neuropeptide Y-immunoreactive neurons were observed in the innermost cell row of the inner nuclear layer (INL). Apart from two cells (Table 2), all NPY-immunoreactive neurons were also GABA-positive (Fig. 1A, B). Although the SP-immunoreactive amacrine cell population is assumed to be homogeneous on the basis of dendritic morphology (HISCOCK and STRAZNICKY, 1989a), it was heterogenous immunohistochemically. Only about one sixth of SP-immunoreactive cells expressed GABA (Table 2), while the rest remained unlabelled for GABA (Fig. 1C, D). Similarly, some of the 5HT-immunoreactive cells expressed GABA (Table 2), while the rest remained unlabelled for GABA (Fig. 1C, D).

<table>
<thead>
<tr>
<th>Antibodies against</th>
<th>Raised in</th>
<th>Source/Reference</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>Rabbit</td>
<td>Sigma</td>
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</tr>
<tr>
<td>GABA</td>
<td>Mouse</td>
<td>Chemicon</td>
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</tr>
<tr>
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<td>Gift from J. FURNESS®</td>
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</tr>
<tr>
<td>Substance P</td>
<td>Rabbit</td>
<td>Amersham</td>
<td>1 : 500</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Rabbit</td>
<td>Incstar</td>
<td>1 : 1200</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Rabbit</td>
<td>Institute J. Boys</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>Phenylalanine hydroxylase</td>
<td>Mouse</td>
<td>Gift from I. TORK®</td>
<td>1 : 1000</td>
</tr>
</tbody>
</table>

### Table 2. Colocalization of GABA-immunoreactive amacrine cells counted in retinal cross sections

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Total number</th>
<th>Double-labeled</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropeptide Y</td>
<td>143</td>
<td>141</td>
<td>98.5</td>
</tr>
<tr>
<td>Substance P</td>
<td>343</td>
<td>55</td>
<td>16.0</td>
</tr>
<tr>
<td>Serotonin</td>
<td>211</td>
<td>65</td>
<td>33.7</td>
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<tr>
<td>Phenylalanine hydroxylase</td>
<td>306</td>
<td>80</td>
<td>23.9</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>102</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Neuropeptide Y-immunoreactive neurons were observed in the innermost cell row of the inner nuclear layer (INL). Apart from two cells (Table 2), all NPY-immunoreactive neurons were also GABA-positive (Fig. 1A, B). Although the SP-immunoreactive amacrine cell population is assumed to be homogeneous on the basis of dendritic morphology (HISCOCK and STRAZNICKY, 1989a), it was heterogeneous immunohistochemically. Only about one sixth of SP-immunoreactive cells expressed GABA (Table 2), while the rest remained unlabelled for GABA (Fig. 1C, D). Similarly, some of the 5HT-immunoreactive cells were double-labelled (Table 2); however, other cells remained unlabelled for GABA (Fig. 2A, B). GABA was not colocalized in any of the TH-immunoreactive amacrine cells (Fig. 2E, F). An anti-phenyl alanine hydroxylase (PH) antibody was utilized for further characterization of 5HT/GABA double-labelled amacrine cells. This antibody has been shown to crossreact with both TH and tryptophan hydroxylase antigens in *Bufo* (ZHU et al., 1992). Since TH-immunoreactive cells did not express GABA in the *Bufo* retina, all neurons double-labeled with anti-GABA and anti-PH antibodies can be considered as 5HT-synthesizing cells. Numerous small-sized PH-immuno-
reactive amacrine cells were seen to be double-labeled for GABA, while large amacrine cells remained single-labeled for PH only (Fig. 2C, D). The ratio of PH/GABA-labeled amacrine cells within the PH-immunoreactive amacrine cell population was smaller than that of the 5HT/GABA double-labeled cells within the 5HT-immunoreactive amacrine cells (Table 2).

**DISCUSSION**

The coexistence of neuropeptides and classical neurotransmitters is well documented in amacrine cell types of various vertebrate species (PouRCHO and GOEBEL, 1988; WATT et al., 1988; CASINI and BRECHA, 1992). Of the two neuropeptides colocalized with GABA in the present study, SP has previously been observed in GABA-immunoreactive amacrine cells of the cat retina (PouRCHO and GOEBEL, 1988). As this study showed that all the SP-immunoreactive cells contain GABA, it is intriguing that the same degree of colocalization could not be observed in the anuran retina. It is possible that the SP-immunoreactive amacrine cells represent neurochemically distinct subpopulations. This notion is supported by the fact that the dendritic coverage of SP-immunoreactive amacrine cells has been estimated at about 6. Thus, these cells can conveniently accommodate neurochemically different subpopulations and still provide a full retinal coverage. Similar observations have been made in the chick retina on the colocalization of enkephalin-immunoreactivity and ³H-GABA and ³H-glycine uptake (WATT et al., 1988), and somatostatin-immunoreactivity and ³H-GABA uptake (LI et al., 1990), where only part of the neuropeptide-immunoreactive cells have been found to accumulate GABA and glycine. Further studies will have to provide
evidence for the presence of glutamic acid decarboxylase in GABA/SP double-labeled cells to exclude the possibility that GABA only leaks into SP-immunoreactive cells through gap junctions, and to determine if other classical transmitters are also colocalized with SP in amacrine cells.

Our report is the first which provides evidence that NPY is colocalized with GABA in a subpopulation of amacrine cells in the anuran retina. It remains to be tested if NPY-immunoreactive amacrine cells, previously reported to be present in the retina of other vertebrates (BRUUN et al., 1986; ISAYAMA and ELDRED, 1988), contain GABA. Since the dendritic arborization pattern of NPY-immunoreactive cells is somewhat different in Bufo (HISCOCK and STRAZNICKY, 1989b) from other vertebrate species (BRUUN et al.,

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**Fig. 2.** Colocalization of GABA-IR in 5HT-IR and -synthesizing and TH-IR amacrine cells. *inl* Inner nuclear layer, *ipl* inner plexiform layer. A and B. 5HT-IR neurons with (arrowheads) and without GABA-IR (arrowheads with asterisks). C and D. Small-sized PH-IR (5HT-synthesizing) amacrine cells exhibit GABA-IR (arrowheads), while large-sized cells do not (arrowheads with asterisks). E and F. A TH-IR amacrine cell (arrowhead with asterisk in E) does not exhibit GABA-IR (arrowhead with asterisk in B). Scale bar in A: 20 μm for all micrographs.
receptor complexes appear to be coupled through a postsynaptic mechanism. GABAB receptor and 5HT1A mechanism could account for the increased efficacy of the substances, such as 5HT and GABA, a similar mechanism of the co-release of classical neurotransmitter sub-
f3-subunit of the GABAA receptor complex. In case which in turn stimulates the phosphorylation of the postulated to be an increase in cyclic AMP-level YEH, 1992). The underlying biochemical mechanism is imposed exposure to this neuropeptide (VERUKI and
scopic current responses are potentiated on super-
cation alone results in no detectable current response (CASINI and BRECHA, 1992). Although VIP applica-
tion alone results in no detectable current response in bipolar and ganglion cells, GABA-activated macro-
scopic current responses are potentiated on super-
posed exposure to this neuropeptide (VERUKI and YEH, 1992). The underlying biochemical mechanism is postulated to be an increase in cyclic AMP-level which in turn stimulates the phosphorylation of the \( \beta \)-subunit of the GABA\(_A\) receptor complex. In case of the co-release of classical neurotransmitter substances, such as 5HT and GABA, a similar mechanism could account for the increased efficacy of the postsynaptic mechanism. GABA\(_B\) receptor and 5HT\(_{1A}\) receptor complexes appear to be coupled through a G-protein to the same potassium channel in the hippocampus (ANDRADE et al., 1986). Revealing the existence of such biochemical mechanisms for the neuroactive substances colocalized in amacrine cells in the anuran retina requires further studies.

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